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Can active immunization redirect an anti-IgE immune response?

Stadler BM, Rudolf MP, Vogel M, Miescher S, Zurcher AW, Kricek F.

Institute of Immunology and Allergology, Inselspital, Bern, Switzerland. stadler@insel.unibe.ch

We used as a template a mouse monoclonal antibody against IgE to isolate peptides from random peptide phage display libraries. Thereby, two types of peptides were isolated that corresponded to two different epitopes on the human IgE molecule. These peptides, also called mimotopes, seem to be a suitable tool in conjunction with carriers to induce an autoimmune response with a beneficial effect in humans, because the originally used template antibody is capable of neutralizing IgE, is nonanaphylactogenic, and inhibits IgE synthesis. The vaccination approach is further supported by the fact that we were capable of isolating anti-idiotypic antibodies from antibody phage display libraries against the template antibody. These anti-idiotypic antibodies were inhibited by both of the isolated IgE mimotopes. Thus, active vaccination with defined IgE mimotopes may represent a follow-up drug for the presently used anti-IgE antibodies.

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Applicant: Pharmacia Diagnostics AB

Inventors: Sabine Flicker, Peter Steinberger, Dietrich Kraft, Rudolf Valenta

GROUP 2 ALLERGEN SPECIFIC IgE-FABS AND USE THEREOF

Field of the invention

The present Invention relates to group 2 allergen specific IgE-Fabs and use thereof. More precisely, the present invention relates to grass pollen specific IgE-Fabs (Phl p2) and reagents, kits and vaccines comprising these. The invention also relates to use of group 2 specific IgE-Fabs for, inter alia, diagnosis, therapy and prevention of type I allergy.

Background of the invention

Almost 500 million individuals suffer from Type I allergy, a genetically determined hypersensitivity disease which is based on the formation of IgE antibodies against *per se* harmless antigens (i. e., allergens) (1). The symptoms of Type allergy (allergic rhinitis, conjunctivitis and allergic asthma) are mainly caused by the crosslinking of effector-cell bound specific IgE antibodies and the consecutive release of biological mediators (e.g., histamine, leukotriens) (2).

Grasses and corn are distributed worldwide, produce large amounts of pollen which become easily airborn and therefore belong to the most important allergen sources. More than 40% of allergic patients are sensitized against grass pollen allergens of which group 2 allergens represent one of the most frequently recognized allergens (3, 4). Group 2 allergens occur in pollen of many grass species as highly homologous proteins with a molecular weight of approximately 10-11 kDa. Approximately 70% of grass pollen allergic people are cross-sensitized to group 2 allergens which is due to sequence, structural, and immunological similarities of group 2 allergens from different grass and corn species (5-7). Recombinant Phl p 2 from timothy grass has been produced as immunologically active recombinant protein which equals the properties of the natural allergen (6). Moreover the three dimensional

structure of rPhl p 2 has been recently determined by NMR analysis and was found to resemble structural features of an immunoglobulin-like domain (8).

Specific therapy of Type I allergy can be achieved in principle by active and passive vaccination. Active vaccination is achieved by specific immunotherapy in order to induce unresponsiveness towards allergens. Although successfully practised since 1911, the immunologically mechanisms of specific immunotherapy are not completely understood (9, 10). Induction of blocking antibodies of the IgG class which interfere with the IgE allergen interaction, modulation of T cell and effector cell responses and generation of tolerance are discussed as possible mechanisms (11). In contrast to active vaccination, passive vaccination is based on the transfer of protective immunoglobulins and represents a routine treatment for many infectious diseases (e.g., hepatitis). The therapeutical efficacy of "passive vaccination" for the treatment of Type I allergy has been demonstrated by classical experiments more than 60 years ago. In 1935 Cooke and collegues reported cure of a hayfever patient by transfer of blood from a patient who had been successfully treated by specific immunotherapy (12). A few years later Loveless showed that the protective effects reported by Cooke are due to blocking antibodies (13). There are several arguments why blocking antibodies may be useful for passive therapy of Type I allergy. First, IgE is the least abundant class of immunoglobulins which could be easily competed. Second, allergic reactions occur in target organs (nose, eyes, lung, skin) where it would be easy to apply blocking antibodies or other competitors of the IgE-allergen interaction avoiding the need of systemic application. The application of the "passive vaccination concept" for treatment of Type I allergy requires however progress in the field of allergen characterization and antibody technology.

Summary of the invention

The present Invention provides human IgE-Fabs suitable for diagnosis and immunotherapy of type I allergy.

In a first aspect, the invention provides group 2 allergen (i.e. pollen allergen from different grass and corn species) specific human IgE Fabs having the amino acid sequences as shown in SEQ ID No 2A-B or essentially homologous variants thereof.

In a second aspect, the invention provides group 2 allergen specific human igE Fabs encoded by the nucleic acid sequences as shown in SEQ ID No 1A-B or essentially homologous variants thereof. For example, variants caused by the degeneracy of the genetic code. The present invention also provides group 2 allergen specific human IgG comprising the variable regions of the above IgE Fabs. Preferably, the whole Ig molecules of the invention are of IgG1 subtype. The present inventors have grafted variable regions of the IgE Fabs of the invention onto human IgG1 using a known vector system. Surprisingly, these complete IgG1 antibodies strongly suppress Phl p2-induced degranulation of patients basophils which indicates their potential for clinical application.

The IgE-Fabs and/or whole Ig according to the invention are directed against Phl p 2, i.e. pollen from timothy grass but cross-react with several other pollen allergens.

The IgE-Fabs and/or whole Ig according to the invention may be recombinantly produced.

In a third aspect, the invention relates to a diagnostic reagent comprising the IgE-Fabs and/or whole Ig according to the invention. Alternatively the reagent comprises the corresponding complete antibodies, and/or modified versions of said Fabs and/or antibodies.

In a fourth aspect, the invention relates to a diagnostic kit comprising the reagent mentioned above. The kit may be any conventional kit for performing an immunoassay.

In a fifth aspect, the invention relates to a vaccine against type I allergy, comprising the IgE-Fabs and/or whole Ig according to the invention.

A preferred use of Phl p 2-specific IgE-Fabs and/or whole Ig and the vaccine according to the invention is for passive immunotherapy of type I allergy. The use may be in preventive or therapeutic purpose.

Another use of Phl p 2-specific IgE-Fabs and/or whole ig according to the invention is for diagnosing of type I allergy.

A further use of Phl p 2-specific IgE-Fabs and/or whole Ig according to the invention is for environmental allergen detection.

Yet a further use of f Phl p 2-specific IgE-Fabs and/or whole Ig according to the invention is for standardization of allergen extracts.

In order to obtain specific competitors of human origin which interfere with the IgE allergen interaction the inventors used the combinatorial library technique (14). An IgE combinatorial library was constructed from lymphocytes of a grass pollen allergic patient (15). The present invention provides human IgE Fabs with specificity for group 2 grass pollen allergens. The human IgE Fabs lack their constant region and therefore cannot activate effector cells to release biologically active mediators. The binding site of the IgE Fabs on group 2 allergens were mapped using recombinant fragments of the major timothy grass pollen allergen Phl p 2 and the crossreactivity of the IgE Fabs with group 2 allergens from different grass and corn species was investigated. Furthermore the Phl p 2-specific IgE Fabs are useful for the identification of group 2 allergen-containing pollen by particle blotting. By ELISA competition experiments, the ability of the IgE Fabs to block binding of grass pollen allergic patients IgE antibodies to Phl p 2 was analyzed. The usefulness of the recombinant group 2-specific IgE Fabs for the detection of environmental allergen loads, the standardization of diagnostic and therapeutic allergen extracts and for passive therapy of grass pollen allergy are discussed.

Detailed description of the invention

MATERIALS AND METHODS

Biological materials, patients sera, antibodies

Pollen from sweet vernal grass, oat, Bermuda grass, rye grass, common reed, Kentucky Bluegrass, rye, wheat and maize were purchased from Allergon (Välinge, Sweden). Sera were collected from grass pollen-allergic patients who were characterized by case history, skin prick testing, RAST, and by testing with recombinant grass pollen allergens as described (16). Sera from non allergic individuals were included as negative controls. ^{125}I-labeled anti-human IgE antibodies (RAST) were obtained from Pharmacia (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden).

E.coli strain XL1-Blue was obtained from Stratagene (La Jolla, CA), and plasmid pComb3H representing a modification of plasmid pComb3 (17) was kindly provided by Carlos F. Barbas, Scripps (LaJolla, CA). Alkaline phosphatase-coupled goat anti-human Fab and goat anti-mouse Fab antisera were purchased from Pierce (Rockford, IL).

Sequence analysis of the cDNAs coding for IgE Fds and light chains

Three phage clones expressing antibody fragments with specificity for Phl p 2 were isolated by panning to rPhl p 2 as described (15). All three clones were checked for the production of Phl p 2-specific Fabs by ELISA and for the correct insertion of cDNAs coding for the heavy chain fragments and the light chains by restriction analysis before sequencing. Plasmid DNA was prepared from recombinant *E. coli* XL-1 Blue using Qiagen tips (Hilden, Germany). Both DNA strands were sequenced using ^{35}S-dCTP (DuPont NEN, Stevenage, UK) and a T7 polymerase sequencing kit (Pharmacia) by primer walking (18). Sequencing primers were obtained from MWG (MWG AG- Biotech, Ebersberg, Germany). The Phl p 2-specific DNA sequences of the heavy chain fragments and the light chains were compared with the germline sequences deposited in the V Base Sequence Directors (Tomlinson *et al.*, MRC Centre for Protein Engineering, Cambridge, UK).

Expression of rPhl p 2-specific Fabs in *E.coli*

E.coli XL1-Blue cells containing the rPhl p 2-specific Fab expressing pComb3H plasmids were grown in SB medium containing 50 µg/ml ampicillin at 37 °C up to O.D. 600nm 0.6-1. After induction with 1.5 mM IPTG, cells were incubated for four hours at 32°C. Induced *E.coli* were harvested by centrifugation at 5000 rpm for 20 min at 4 °C and supernatants were stored at –20 °C for ELISA analysis. Cells were resuspended in PBS containing 1 mM PMSF, homogenized with an ultraturrax (Ika, Stauffen, Germany) and centrifuged at 18,000 rpm for 20 min at 4 °C (extract). The extracts were tested in ELISA assays for their binding capacity to rPhl p 2 as described (15).

Expression of recombinant fragments of the Phl p 2 allergen for mapping of the binding sites of the human IgE Fabs

Lambda gt11 phage clones expressing ß-galactosidase fused complete Phl p 2 and Phl p 2 fragments were generated as described (8). *E.coli* Y1090 were plated as confluent lawn onto LB plates containing 100mg/l ampicillin (19). Two µl aliquots of phage lysates containing > 10^6 plaque-forming units of each phage clone were dotted onto the *E. coli* lawn in defined order and plates were incubated at 43°C until plaques became visible. The synthesis of recombinant protein was induced by overlay with 10 mM IPTG-soaked nitrocellulose filters (Schleicher& Schuell, Dassel, Germany) for additional 4 hours. The clones which reacted with the IgE Fabs were identified by immunoscreening. For this purpose filters were washed twice for 5 min and once for 30 min with TBST containing 0.5% w/v BSA at roomtemperature and then probed overnight with *E. coli* extracts containing anti-Phl p 2 Fab and, for control purposes, with extracts from *E. coli* transformed with the empty pComb 3H plasmid at 4 °C. After washing, the Fabs were detected with an alkaline phospatase-coupled goat anti-human Fab antiserum diluted 1:5000 in TBST/0.5% w/v BSA.

Preparation of natural pollen xtracts; identification of gr up 2 allergens by immunoblotting

Hundred mg of pollen of each grass and corn species were resuspended separately in 5 ml SDS-sample buffer and homogenized with an ultraturrax (IKA, Stauffen, Germany) for 1 min, boiled for 5 min at 95 °C and centrifuged with 14500 rpm for 5 min to remove insoluble materials. Comparable amounts of protein extracts (100 µg/slot) were then separated by a 14% SDS-PAGE under reducing conditions, stained with Coomassie Brillant Blue or blotted onto nitrocellulose (Schleicher & Schuell) (20-22).

Nitrocellulose membranes containing blotted pollen extracts from the different grass and corn species were blocked twice for 5 min and once for 30 min with TBST containing 0.5% w/v BSA at roomtemperature and then exposed overnight with bacterial extracts containing Phl p 2-specific IgE Fabs at 4 °C. After washing, membranes were incubated with an alkaline phospatase-coupled goat anti-human Fab antiserum diluted 1:5000 in TBST/0.5% w/v BSA, respectively to detect bound Fabs.

Particle blotting

Pollen grains from sweet vernal grass, oat, Bermuda grass, rye grass, timothy grass, common reed, Kentucky Bluegrass, rye, wheat, maize and birch (control) were applied to a nitrocellulose membrane in defined order using toothpicks. Membrans were placed on water-soaked Whatman paper for half an hour to allow the release of allergens. Released proteins were detected with Ponceau S (Boehringer, Mannheim, Germany) as described (19). Group 2 allergens were detected with Phl p 2-specific IgE Fabsas described for the immunoblotting. For the identification of pollen grains releasing the major birch pollen allergen Bet v 1 a recombinant mouse anti-Bet v 1 Fab was used which was detected with an alkaline phosphatase-conjugated goat anti-mouse Fab antiserum (Pierce).

IgE competition experiments

The ability of Phl p 2-specific IgE Fabs to inhibit the binding of grass pollen allergic patients IgE antibodies to Phl p 2 was studied by immunoblot competition experiments. Nitrocellulose strips containing equal aliquots of blotted rPhl p 2 (1µg/cm) were blocked twice for 5 min and once for 30 min in buffer A (50 mM sodium phosphate buffer, pH 7.4, containing 0.5% w/v BSA, 0.5% v/v Tween 20 and 0.05% w/v NaN_3) at roomtemperature and then preincubated overnight with bacterial extracts containing Phl p 2 specific IgE Fabs and, for control purposes, with bacterial extracts containing an anti-Phl p 5 IgE Fabs (both at a concentration 5µg/ml) at 4 °C. After washing, strips were incubated overnight with sera from grass pollen allergic patients diluted 1: 5 in buffer A at 4°C. Bound IgE antibodies were detected with ^{125}I-labeled anti-human IgE antibodies (RAST, Pharmacia) and the amount of bound IgE antibodies was quantified by gamma-counting in a gamma counter (1277 Gammamaster, LKB, Wallac). All experiments were performed as duplicates with variations of less than 10% and results represent mean values. The percentage inhibition of IgE binding was calculated from the mean cpm value measured with or without addition of competing Fab. Percentage inhibition = 100-cpm_{Fab} x 100/$cpm_{control}$.

RESULTS

The results will be discussed below in association with the accompanying sequence listing and drawings:

SEQ ID NO 1. DNA sequence comparison of the IgE Fabs. SEQ ID NO 1A shows the alignment of the clone 94 heavy chain DNA sequence with those of clones 60 and 100. SEQ ID NO 1B displays the sequence alignment of the three light chain cDNAs. The Xho I and the Sac I sites are printed in italics. Framework (FR1-FR4) and hypervariable (CDR1-CDR3) regions are labeled. Identical amino acids are indicated by dashes.

SEQ ID NO 2. Amino acid sequence alignment. SEQ ID NO 2A shows the alignment of the heavy chain amino acid sequences derived from three Phl p 2-specific IgE Fabs (clones 60, 94, 100) and that of the heavy chain of a homologous human IgM rheumatoid factor (accession number: Y14936). SEQ ID NO 2B displays the amino acid sequence alignment of the IgE Fab-derived light chains and three homologous light chains from an anti-Rh (D) antibody (AF044462) and two rheumatoid factors (S56199, S67059). The framework (FR1-FR4) and hypervariable (CDR1-CDR3) regions are labeled and identical amino acids are indicated by dashes.

Figure 1. Comparision of the variable heavy chain (Fig. 1A) and light chain (Fig. 1B) DNA sequence of clone 94 with the closest related germline sequences (accession numbers are printed on the left margins). The positions of the CDRs and FRs are indicated. Nucleotides are grouped in aa-encoding tripletts and identical nucleotides are displayed by dashes.

Figure 2. Mapping of the binding site of the Phl p 2-specific human IgE Fab on the Phl p 2 allergen. Overview of the recombinant Phl p 2 fragments comprising the N-terminal 64 amino acids and the C-terminal 69 amino acids (Figure 2A). Figure 2B shows a ribbon representation of the three-dimensional structure of the Phl p 2 allergen in which the N-terminal and C-terminal fragment has been indicated. Figure 2C: Binding of the clone 94-derived IgE Fab to nitrocellulose filters containing complete rPhl p 2 (1), the N-terminal (2), C-terminal (3) Phl p 2-derived fragments and ß-gal alone (4). For control purposes, filter-bound clones were exposed to bacterial extracts from *E. coli* transformed with empty plasmid (pComb3H).

Figure 3. rPhl p 2-specific IgE-Fabs crossreact with natural group 2 allergens from different grasses. rPhl p 2-specific IgE Fabs were tested for reactivity with nitrocellulose-blotted grass pollen extracts (Ant o: sweet vernal grass; Ave s: oat; Cyn d: Bermuda grass; Lol p: rye grass; Phr c: common reed; Poa p: Kentucky Blue grass; Sec c: rye; Tri s: wheat; Zea m:

maize, Phl p: timothy grass). The position of the 14.3 kDa marker is indicated with an arrow on the right margin.

Figure 4. Detection of pollen grains containing group 2 allergens by particle blotting. Pollen grains from ten different monocots (*Ant o*: sweet vernal grass; *Ave s*: oat; *Cyn d*: bermuda grass; *Lol p*: rye grass; *Phl p*: timothy grass; *Phr c*: common reed; *Poa p*: Kentucky Blue grass; *Sec c*: rye; *Tri s*: wheat; *Zea m*: maize) and birch (*Bet v*) were dotted to nitrocellulose. Released proteins were stained with Ponceau S (A). Group 2 allergens were detected with Phl p 2-specific IgE Fabs (B), the major birch pollen allergen, Bet v 1, with specific Fabs (C).

The three Phl p 2-specific IgE Fabs contain closely related heavy chain fragments which have recombined with different light chains: sequence homology with autoantibodies

Framework as well as complementarity determining regions of the three heavy chain fragments (clones 94, 60, 100) were of equal size. (SEQ ID NO. 1A) and their VH regions showed the highest similarity with members of the VH4 family (e. g., accession number: U71106; 23). The alignment of the cDNAs coding for the heavy chain variable regions of the three clones shows that they differ only in few nucleotides (27 out of 342 bp, 8% for clone 94 versus clone 60; 18 out of 342 bp, 5% for clone 94 versus clone 100; 9 out of 342 bp, 3% for clone 60 versus clone 100). The nucleotide exchanges were equally distributed over the complete variable region including frameworks and CDRs (SEQ ID NO. 1A).

Sequence analysis of the light chain cDNAs revealed that they all belonged to the kappa family. The sequence comparison of the three light chains showed much greater variation than was observed among the heavy chains (SEQ ID NO. 1B). Most of the nucleotide exchanges were observed in the CDR1 (9%-33%) and in the CDR3 (25%-33%).

A comparision of the deduced amino acid sequences of the variable regions of the heavy chain fragments of the three rPhl p 2-specific IgE Fab clones is displayed in SEQ ID NO 2A. Clones 60 and 100 are very similar (95% sequence identity) whereas a more moderate sequence identity of 86% and 88% was observed between clones 60 and 94, and

between clones 94 and 100, respectively. The few amino acid exchanges were sometimes not conservative ones and equally distributed over the framework and complementarity determining regions of the three clones (Fig. 2A). When compared with known human variable regions a surprising sequence similarity was found to a human IgM rheumatoid factor (accession number: Y14936; 24) (Fig. 2A) With exception of the CDR3 region which was completely different in sequence and length between the heavy chain fragments of the IgE Fabs and the rheumatoid factor, a comparable sequence identity was found for CDR1 and CDR2 as well as for all 4 framework regions of the IgE Fabs and the rheumatoid factor (SEQ ID NO 2A).

SEQ ID NO. 2B shows the alignment of the deduced amino acid sequences of the light chains of the three clones. The amino acid sequences of the three light chains showed a considerable sequence variation, particularly in the CDR1 (27-46%) and in the CDR3 (56%). The CDR2 regions differed only in one or two aa exchanges and also in framework regions only few amino acid exchanges were noted (<10% in FRW1, <14% in FRW2, <10% in FRW3) (SEQ ID NO 2B).

The three IgE Fab-derived light chains showed an interesting amino acid sequence similarity with light chains from human autoantibodies, one from a human anti-Rh (D) antibody (accession number: AF044462; 25) and two human rheumatoid factors (accession numbers S56199; S67059; 26, 27).

Somatic mutations in the variable regions of the IgE Fabs are indicative of an antigen-driven selection process

Fig. 1A shows the comparision of the DNA sequence of the variable region of the clone 94 heavy chain fragment with the closest related germline sequences (accession number: Z12365: V-genes, FR1- FR3; X97051: D-genes, CDR3; X86355: J-genes, CDR3+ FR4) (22-24). In total we found 26 nucleotide exchanges of which 11% (10/93) were found in the CDRs and 7% (16/243) were observed in the framework regions. Five of these mutations were silent whereas 18 mutations led to amino acid exchanges.

The comparision of the variable region DNA sequence of the clone 94 light chain with the most closely related germline sequences (accession number: X93627: V-genes, FR1-CDR3; J00242: CDR3+ FR4; 25, 26) is shown in Figure 1B. We found 18 nucleotide exchanges of which 11% (9/81) were located in the CDRs and 4% (9/228) occured in the frame work regions. Most mutations were found in the CDR3.

A recombinant fragment comprising the N-terminal 64 amino acids of Phl p 2 contains the binding site for the IgE Fabs

In order to determine the binding site for the rPhl p 2-specific IgE Fabs, nitrocellulose-dotted complete ß-gal-fused rPhl p 2 allergen (96 aa), a N-terminal rPhl p 2 fragment comprising the first 64 aa portion (1-64) and a 69 aa long C-terminal rPhl p 2 fragment (28-96 aa) were exposed to the Fabs (Fig. 2A). Fig. 2B shows a ribbon representation of the complete Phl p 2 allergen which is similar to an immunoglobulin domain and consists mainly of ß-sheet structure. The N-terminal and C-terminal fragment have been indicated in the model (Fig. 2B). These two fragments were previously identified as IgE-reactive domains whereas smaller fragments did not show IgE binding capacity (8). As exemplified for clone 94, all three Phl p 2-specific IgE Fabs recognized the complete rPhl p 2 (1) and the N-terminal rPhl p 2 fragment (2) but did not bind to the C-terminal fragment (3) nor to λgt 11 phage control proteins (4) (Fig. 2C). Bacterial extracts obtained from bacteria which were transformed with the empty pComb3H plasmid did not react with any of the dotted proteins (Fig. 2C). No reactivity of the Phl p 2-specific IgE Fabs with the smaller Phl p 2 fragments described previously (8) was observed (data not shown).

Anti-Phl p 2 IgE Fabs crossreact with natural group 2 allergens from 7 grass and corn species and identify group 2 allergen-containing pollen by particle blotting

In order to investigate whether the recombinant human IgE Fabs crossreacted with natural group 2 allergens from different grass and corn species, nitrocellulose-blotted natural pollen extracts from several grass and corn species were tested (Fig. 3). The IgE Fabs

reacted strongly with group 2 allergens in sweet vernal grass, rye grass, Kentucky Bluegrass, rye and wheat. Only weak reactivity was observed with oat and common reed. No reactivity was seen with pollen extracts from monocots reportedly lacking or containing low levels of group 2 allergens (Bermuda grass, maize) (16).

Environmental measurements of pollen allergen loads are currently performed by air sampling and analysis of pollen morphology. We demonstrate that Phl p 2-specific Fabs can be used for the immunological identification of pollen grains containing group 2 allergens by particle blotting (Fig. 4). Membran-bound pollen grains from ten different grass species and birch released proteins after hydration which were stained with Ponceau S (Fig. 4A). Phl p 2-specific Fabs identified exclusively grass pollen grains containing group 2 allergens (sweet vernal grass, rye grass, Kentucky Bluegrass, rye, wheat and oat) (Fig. 4B). A mouse Fab with specificity for the major birch pollen allergen, Bet v 1, reacted only with birch pollen confirming the specificity of the assay (Fig. 4C).

Phl p 2-specific IgE Fabs inhibit binding of grass pollen allergic patients IgE antibodies to Phl p 2

Table 1 displays the quantification of IgE binding of sera from 27 grass pollen allergic patients to nitrocellulose-blotted rPhl p 2 which was preincubated with Phl p 2-specific IgE Fabs versus preincubation with Phl p 5-specific IgE Fabs (control) (Table 1). In 14 sera (52%) preincubation with Phl p 2-specific IgE Fabs reduced serum IgE binding > 50% (Table 1). In 10 sera (38%) the decrease of IgE binding was between 30-50% and in the remaining 3 sera (10%) a reduction of IgE binding between 22-29% could be achieved (Table 1). An average inhibition of IgE binding to Phl p 2 in range of 52% was achieved when Phl p 2 was preincubated with Phl p 2-specific IgE Fabs (Table 1).

Table 1. IgE Fabs inhibit the binding of allergic patients' IgE to rPhl p 2. Sera from 27 grass pollen allergic patients (#1-27) were exposed to nitrocellulose-blotted rPhl p 2 which has been preincubated with Phl p 2-specific IgE Fabs (column A) or with control proteins (column B). The cpm values corresponding to the amount of bound IgE antibodies are displayed in columns A and B. The third column shows the percentage of inhibition of IgE binding. The mean inhibition of IgE binding is displayed at the bottom of the third column.

Patient No.	cpm: with preincubation with Phl p 2-specific Fab	cpm: without preincubation with Phl p 2-specific Fab	% of reduction
1	859.2	1389.4	38
2	406.5	2570.5	84
3	93.3	222.7	58
4	150.7	228.7	34
5	734.1	1222.8	40
6	895.5	4240	79
7	6482.3	17914.2	64
8	130.8	253.1	48
9	293.5	523.5	44
10	1508.4	4274.9	65
11	2238	4520.1	50
12	1088.5	1779.7	38
13	855.9	1423.7	40
14	194.6	313	38
15	252.4	1014.6	75
16	312.5	448.4	30
17	1350.3	3505.3	61
18	140.4	387.6	64
19	1343.3	3485.5	61
20	129	416.4	69
21	704.4	1161.7	39
22	122.5	173.5	29
23	1707.4	2360.6	28
24	600.6	2036.8	70
25	3495	4470.6	22
26	1128.9	3634.8	69
27	426.4	1323.2	68
			mean: 52

DISCUSSION

The present inventors report the molecular and immunological characterization of three human IgE antibody fragments with specificity for one of the most important environmental allergens, the major timothy grass pollen allergen, Phl p 2. The IgE Fabs were isolated from an IgE combinatorial library which was constructed from lymphocytes of a grass pollen allergic patient via panning to recombinant Phl p 2. Sequence analysis revealed that all three heavy chain fragments belonged to the VH4 family and were closely related to each other (23). While in the heavy chain variable regions the few amino acid sequence variations were equally distributed over the framework and complementarity determining regions, the kappa light chains were less closely related and showed significant sequence variations in the CDR1 and CDR3 domains. We have already previously found that IgE Fabs with specificity for the major timothy grass pollen allergen consisted of identical heavy chain fragments which had recombined with different light chains (15). Furthermore it has been demonstrated that the Phl p 5-specific IgE heavy chain fragments can be recombined with light chains from antibodies with different specificity but retain antigen-specificity (15, Laffer and Valenta, unpublished data). Also our study indicates that similar heavy chain fragments can recombine with different light chains but retain specificity for the original allergen. Although we have no proof that exactly those heavy chain and light chain combinations which we isolated via the combinatorial library approach existed in the allergic patient used for construction of the library, the latter findings support the assumption that the specificity of certain IgE antibodies is dictated by the heavy chains and that promiscuous use of light chains is possible without loosing allergen specificity. In this context it is of note that all three IgE Fabs bound to the same recombinant fragment comprising the N-terminal 64 amino acids of Phl p 2.

The close sequence similarity of the heavy chain fragments as well as of the light chains of the Phl p 2-specific IgE Fabs with human autoantibodies (rheumatoid factors, anti Rh (D) antibodies) is of note in view of the fact that the recently determined three dimensional structure of Phl p 2 exhibited an unexpected similarity with immunoglobulin-like

domains occuring in man (8). So far we have no evidence that the IgE Fabs can react with human proteins but when tested with pollen extracts from a variety of grass and corn species we found extensive crossreactivity with group 2 allergens. The recombinant Phl p 2-specific IgE Fabs may therefore be used to determine loads of group 2 allergens in the environment and may thus be useful for preventive measures. Furthermore they may be used for the determination and standardization of group 2 allergens in natural allergen extracts currently used for diagnosis and treatment of grass pollen allergy. Our finding that Phl p 2-specific IgE Fabs reacted with a major epitope-containing domain of Phl p 2 and strongly (mean inhibition 52%) inhibited binding of grass pollen allergic patients (n=27) complete IgE antibodies to Phl p 2 indicates a therapeutic potential of the Fabs. If the Fabs can be attached to epithelial cells or antigen-presenting cells in the target organs of atopy it may even become possible to build up a stable defense line against intruding allergens and perhaps to target them directly into the proteolytic compartment of antigen presenting cells without activating effector cells. The latter approach may not only inactivate the allergen via proteolysis but may be also useful for the induction of a protective mucosal immunity.

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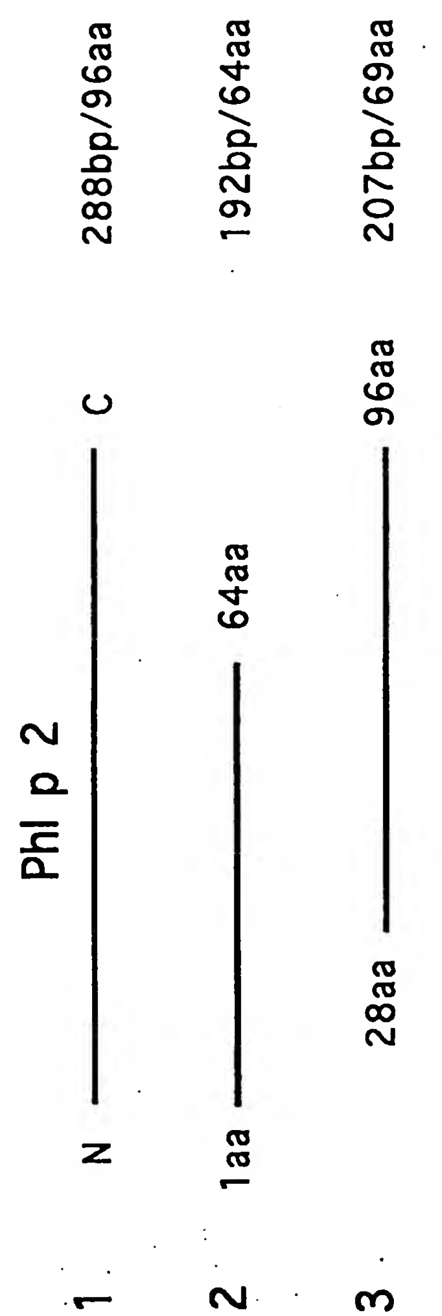
Fig. 1A

```
                                               FR1
94        TCT GGC CCA GGA CTG GTG AAG CCT GCA CAG ACC CTG TCC CTC AGC TGC GCT GTC TCT GGC
Z12365    --G --- --- --- --- --- --- --- T-- --- --- --- --- --- -C- --- A-- --- --- --T

             FR1                        CDR1                          FR2
94        GGC TCC ATC CGC   AGT GGT GGT TAC TAC TGG AGT   TGG ATC CGC CAA CAC CCA GGG AAG
Z12365    --- --- --- A--   --- --- --- --- --- --- --C   --- --- --- --G --- --- --- ---

                FR2                                CDR2
94        GGC CTG GAG TGG ATT GGG    TAC ATC TAT CAC AGT GGG AAC ACC TAC TAC AAC CCG TCC
Z12365    --- --- --- --- --- ---    --- --- --- T-- --- --- -G- --- --- --- --- --- ---

             CDR2                                FR3
94        CTC AAG AGT    CGA ATT GCC ATG TCG GTA GAC ACG TCT GAG AAC AAG TTC TCC CTG AGG
Z12365    --- --- ---    --- G-- A-- --A --A --- --- --- --- A-- --- C-- --- --- --- -A-

                                               FR3
94        CTG AAC TCT GTG ACT GCC GCG GAC ACG GCC GTG TAT TAC TGT GCG AGG
Z12365    --- -G- --- --- --- --- --- --- --- --- --- --- --- --- --- --A

                 CDR3
94        TTA GAT GGC TAC ACT TTG
X97051    AG- --- --- --- -A- -AC

           CDR3                    FR4
94        GAC ATC   TGG GGC CAG GGA ACC CTG GTC ACC GTC TCC TCA
X86355    --- TA-   --- --- --- --- --- --- --- --- --- --- ---
```

Fig. 1B

```
                                          FR1
94       ACT CAG TCT CCA TCC TCC CTG TCT GCA TCT GTG GGA GAC AGA GTC ACC ATC AGT TGC
X93627   --C --- --- --- --- --- --- --- --- --- --A --- --- --- --- --- --- -C- ---

                   CDR1                                                  FR2
94       CGG GCA AGT CAG AGA ATT AAC ACC TAT TTA AAT     TGG TAT CAG CAT AAA CCA GGG
X93627   --- --- --- --- --C --- -G- -G- --- --- ---     --- --- --- --G --- --- ---

                   FR2                                  CDR2                   FR3
94       AAA GCC CCT AAG CTC CTG ATC TAT     GCT GCA TCC AGT TTG CAA AGT     GGG GTC
X93627   --- --- --- --- --- --- --- ---     --- --- --- --- --- --- ---     --- ---

                                          FR3
94       CCA TCA AGG TTC AGT GGC AGT GGA TAT GGG ACA GAC TTC ACT CTC ACC ATC AGC AGT
X93627   --- --- --- --- --- --- --- --- -C- --- --- --T --- --- --- --- --- --- ---

                                  FR3                                 CDR3
94       CTG CAG CCT GAA GAT TTT GCA AGT TAC TAC TGT     CAA GAG AGT CTC AGT GCC TCG
X93627   --- --A --- --- --- --- --- -C- --- --- ---     --- C-- --- TA- --- A-- CT-

            CDR3                      FR4
94       TAC ACT   TTT GGC CAG GGG ACC AAG GTG GAG ATC AAA CGA
J00242   --- ---   --- --- --- --- --- --- C-- --- --- --- ---
```

Fig. 2A


Phl p 2
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N
C
288bp/96aa
2
1aa
64aa
192bp/64aa
3
28aa
96aa
207bp/69aa

Fig. 2B

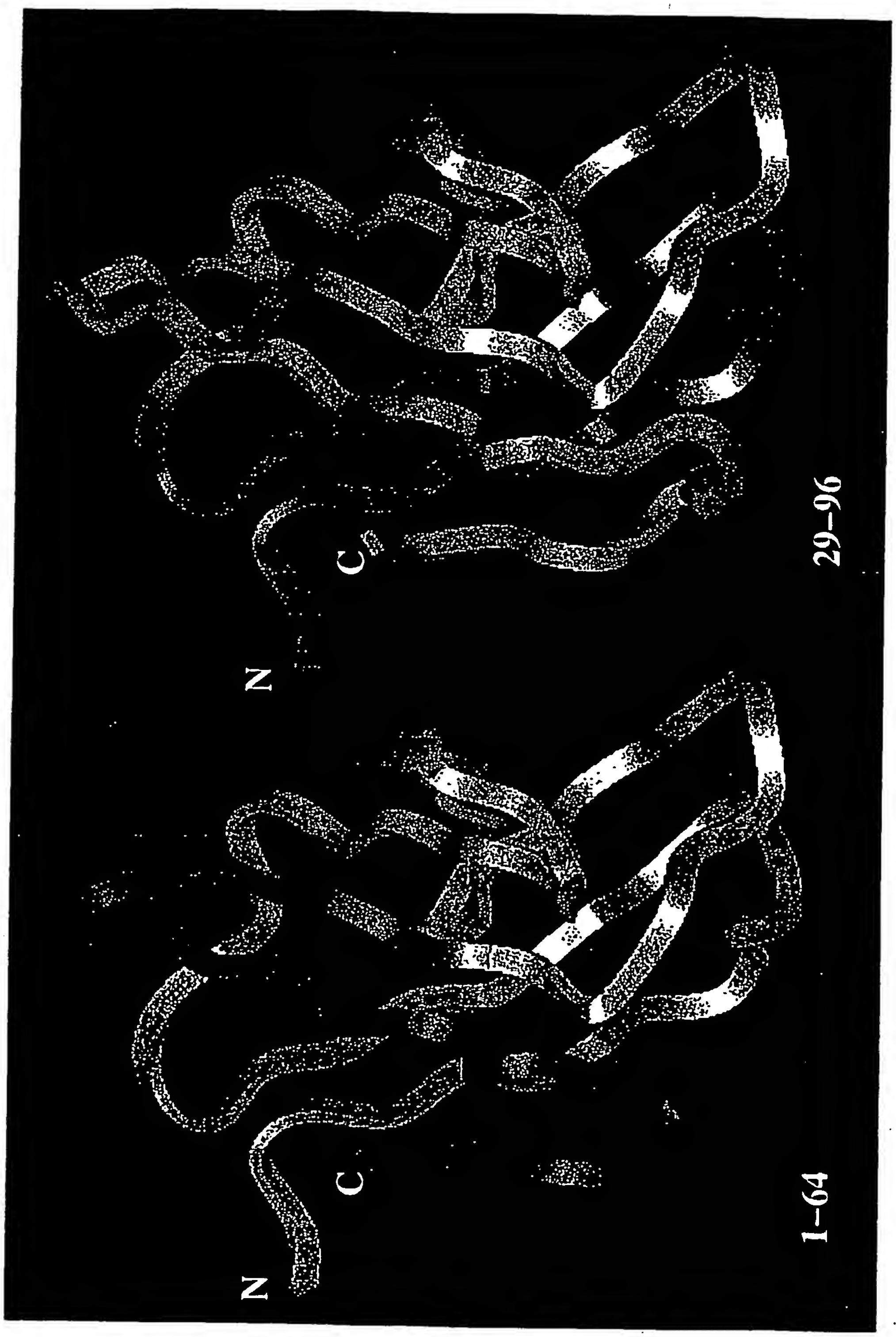

Fig. 2C

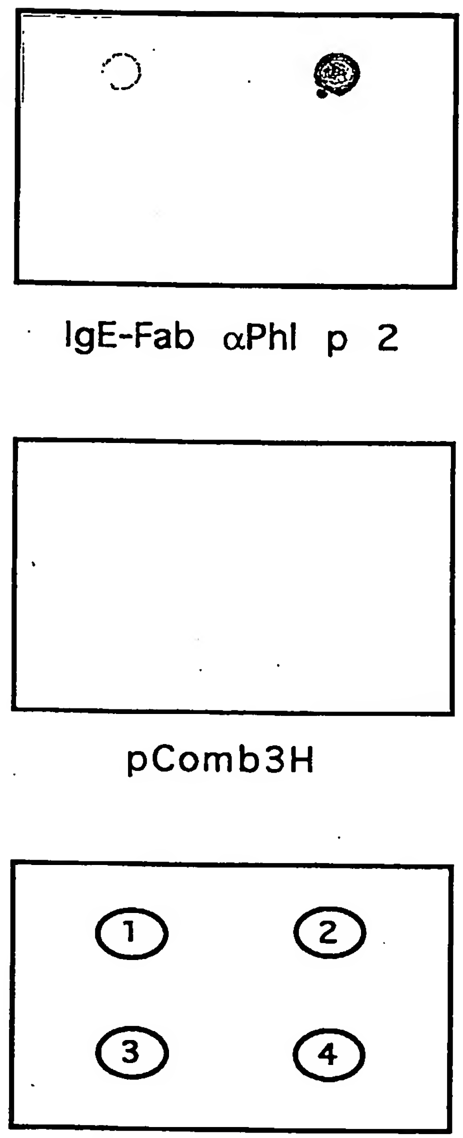

Fig. 3

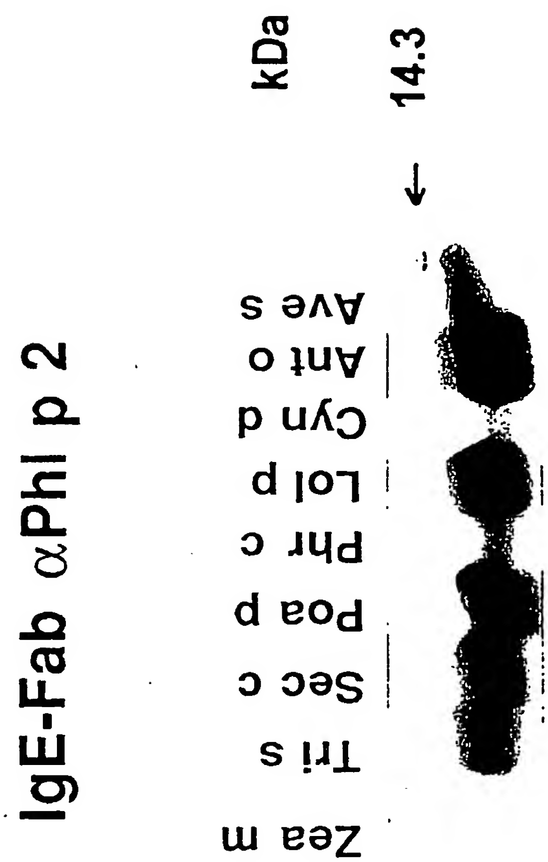
IgE-Fab αPhl p 2
kDa
14.3
Ave s
Ant o
Cyn d
Lol p
Phr c
Poa p
Sec c
Tri s
Zea m

Fig. 4